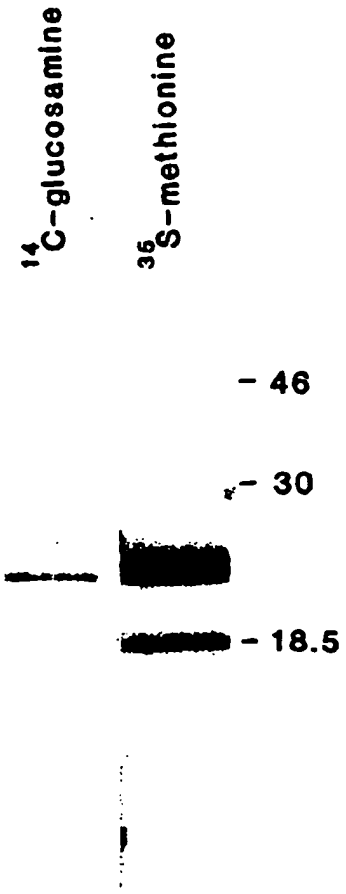

b

in CHO cells. (a) CHO cells transfected
methionine, 100 μCi/ml, 1164 Ci/mmol as
with anti-IFN-β antibodies, and immuno-
culture represents data from wild-type
copies of pM17 were superinduced and
onine as indicated; the supernatants were
analyzed on SDS–PAGE.

side, 10% ethylene glycol in loading buffer) is added to the column; after 15 min on the column, material is collected, dialyzed against PBS, and titrated for IFN activity.

Concluding Comments

We have applied the techniques described above to establish CHO cell lines that can be induced to secrete glycosylated and unglycosylated forms of human IFN-β. While initial levels can be low, about 100 units/ml, we have been able to coamplify the IFN-β gene along with the DHFR gene by methotrexate selection and to obtain very high levels of IFN expression in selected cell lines.[5] Immunoprecipitates of [^{35}S]methionine-labeled supernatants from initial transfectants of CHO cells expressing wild-type IFN-β (pM17) or a mutant IFN-β (pMB-1) gene where glutamine was substituted for asparagine at position 80 are shown in Fig. 1a. Cells expressing the modified IFN-β (pMB-1) secrete a form of IFN-β that migrates at 18,500 D on SDS–PAGE, and do not produce the major 23,000 form seen in supernatants from cells expressing unmodified IFN-β (pM17). An 18,500 D form of IFN-β is also produced from the wild-type gene and appears to be unglycosylated, as shown in Fig. 1b. The 18,500 D form of IFN-β produced from the pMB-1 gene appears to be unglycosylated confirming that the asparagine residue at position 80 is the site for glycosylation in native IFN-β. Preliminary results indicate that the unglycosylated IFN-β produced and secreted from the mutant gene has a much lower specific biological activity than glycosylated IFN-β. The availability of these cell lines will permit us to evaluate further the role of glycosylation in the activity and physical properties of IFN-β.

[58] Procedures for *in Vitro* DNA Mutagenesis of Human Leukocyte Interferon Sequences

By THOMAS M. DECHIARA, FRAN ERLITZ, and S. JOSEPH TARNOWSKI

Introduction

The mutagenesis of cloned genes has become a powerful research tool in the analysis of protein function (for a review, see Dalbadie-McFarland and Richards[1]). This chapter describes the production of recombinant

[1] G. Dalbadie-McFarland and J. H. Richards, *Annu. Rep. Med. Chem.* **18**, 237 (1983).

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human leukocyte interferon A (IFN-αA) analogs to understand better how the structure of IFN-αA is related to its biological activity. Two approaches were taken to specifically mutate the IFN-αA gene. In one approach, synthetic deoxyoligonucleotides were employed to replace a desired region of IFN-αA DNA located between convenient restriction enzyme recognition sites. These deoxyoligonucleotides were identical in sequence to the segment they replaced, except for a single codon change. The mutated genes encoded the substitution of cysteine residues 1 and 98 in the protein with glycine and serine, respectively. In the second approach, site-specific mutations were directed by single deoxyoligonucleotides in a procedure which utilized a heteroduplex of plasmid DNA. These mutations were in the IFN-αA gene region which encoded the carboxy terminal 27 amino acids.

Solution and Materials

TAE: 40 m*M* Tris-acetate, pH 7.8, 5 m*M* NaOAc, 2 m*M* EDTA
TE: 10 m*M* Tris · HCl, pH 7.4, 1 m*M* EDTA
Ligation solution: 60 m*M* Tris · HCl, pH 7.5, 10 m*M* $MgCl_2$, 10 m*M* dithiothreitol (DTT), 400 μ*M* ATP. All ligation reactions were performed at 15° for 12 hr in a volume of 10 μl except where indicated.
Restriction endonucleases: *Bst*EII, *Eco*RI, *Hin*fI, *Pvu*II, and *Sau*3AI were from New England Biolabs. The buffer conditions used were described by the manufacturer.
Plasmids: (1) pRC23 is described in detail in this volume.[2] (2) pRC234 is a derivative of pRC23 in which the *Pvu*II restriction endonuclease site was deleted by a brief *Bal*31 exonuclease digestion followed by ligation of the blunt ends to recircularize the plasmid. The IFN-αA gene was inserted into the unique *Eco*RI site of these vectors.
Klenow fragment of *E. coli* DNA Polymerase I and T_4-DNA ligase from Boehringer Manheim.

Substitution of Cys 1 and Cys 98 Residues

The nucleotide sequence of the IFN-αA DNA[3] predicts cysteine residues at positions 1, 29, 98, and 138 in the protein (Fig. 1a). Disulfide bond assignments,[4] together with selective reduction studies,[5] have suggested

[2] R. Crowl, this volume [55].
[3] S. Pestka, *Arch. Biochem. Biophys.* **221**, 1 (1983).
[4] R. Wetzel, *Nature (London)* **289**, 606 (1981).
[5] R. Wetzel, H. L. Levine, D. A. Estell, and S. Shire, *J. Cell. Biochem., Suppl.* **6**, 89 (1982).

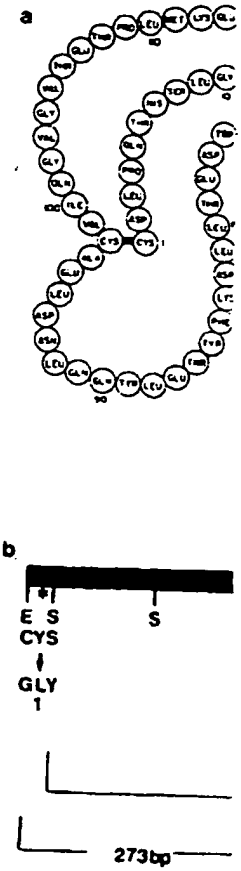

FIG. 1. (a) Schematic representa
The gene encoding IFN-αA is carri
about 500 bp of coding sequences
DNA. E. *Eco*RI; S, *Sau*3AI; P, *Pv*

that, in contrast to the Cys
bond is less stable and is no
αA as measured *in vitro*. The
98 residues, or both, by clos
on the biological activity of
(Table I) were used to repl
gene between the *Eco*RI and
of Cys 1, or between the *Pv*

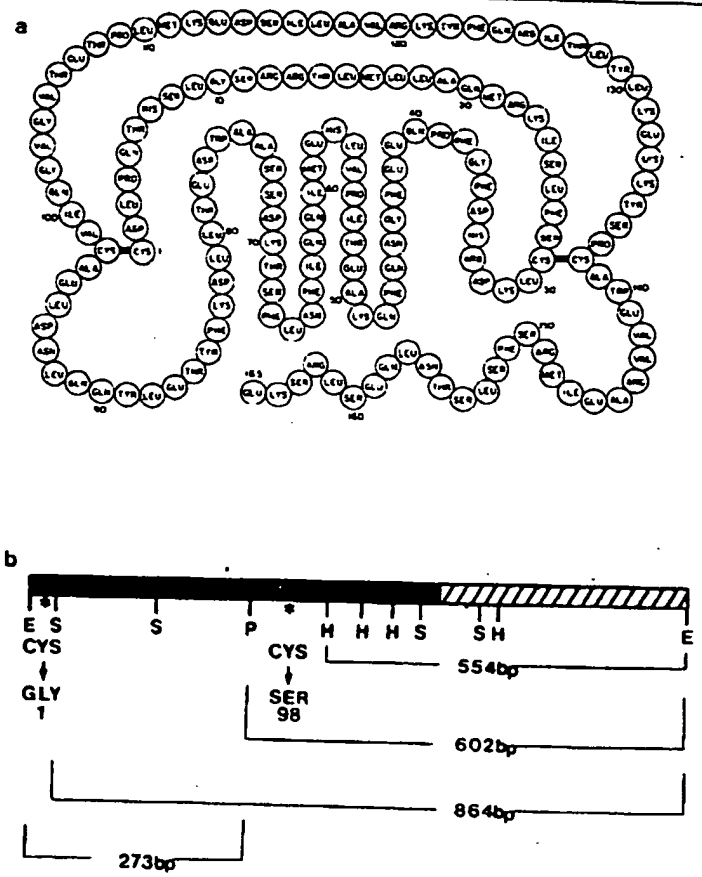

FIG. 1. (a) Schematic representation of IFN-αA (taken with permission from Pestka[3]). (b) The gene encoding IFN-αA is carried on an *Eco*RI fragment.[6,7] The solid region represents about 500 bp of coding sequences and the hatched region, about 350 bp of 3' untranslated DNA. E, *Eco*RI; S, *Sau*3AI; P, *Pvu*II; H, *Hin*fI.

that, in contrast to the Cys 29/Cys 138 disulfide bond, the Cys 1/Cys 98 bond is less stable and is not a requisite for the antiviral activity of IFN-αA as measured *in vitro*. Therefore, substitution of either the Cys 1 or Cys 98 residues, or both, by closely related amino acids should have no effect on the biological activity of the protein. Synthetic deoxyoligonucleotides (Table I) were used to replace the amino terminal coding region of the gene between the *Eco*RI and *Sau*3AI restriction sites for the substitution of Cys 1, or between the *Pvu*II and *Hin*fI sites to substitute for Cys 98

TABLE I
SYNTHETIC DNA TO REPLACE PARENTAL IFN-αA GENE FRAGMENTS[a]

Synthetic deoxyoligonucleotide	Sequence
	1
	Gly
1A (11-mer)	AATTCATGGGC ... *Sau*3AI
1B (11-mer)	*Eco*RI ... GTACCCGCTAG
	*Pvu*II
98A (18-mer)	CTGAATGACCTGGAAGCC
98B (27-mer)	GACTTACTGGACCTTCGGTCGCACTAT
	98
	Ser
98C (28-mer)	AGCGTGATACAGGGGGTGGGGGTGACAG ... *Hin*fI
98D (22-mer)	GTCCCCCACCCCCACTGTCTGA

[a] Dashed line, restriction site overhang or blunt end; solid line, 9 base complementary sequence for the ligation of 98A, B, C, and D.

(Fig. 1b).[6,7] Each set of synthetic DNA molecules encoded one codon change. The TGT codon for Cys in the parental gene was replaced by GGC (Gly) or by AGC (Ser) where indicated (Table I).

Three mutated IFN-αA genes were constructed: one encoding the Gly 1 substitution, one encoding Ser 98, and one encoding both. The incorporation of glycine in the first amino acid position was achieved by the ligation of a 50-fold molar excess of synthetic deoxyoligonucleotides 1A and 1B (Table I) to the 864 bp *Sau*3AI/*Eco*RI gene fragment. This fragment was generated by *Sau*3AI partial digestion of IFN-αA DNA, necessitated by multiple *Sau*3AI restriction sites (Fig. 1b). Mutagenesis for the incorporation of Ser 98 resulted from the ligation of a 50-fold molar excess of deoxyoligonucleotides 98A, 98B, 98C, and 98D (Table I) to both the 273 bp *Eco*RI/*Pvu*II fragment and the 554 bp *Hin*fI/*Eco*RI fragment. The larger fragment was generated by *Hin*fI partial digestion of the 602 bp *Pvu*II/*Eco*RI gene fragment (Fig. 1b). The double substitution of Gly 1

[6] S. Maeda, R. McCandliss, M. Gross, A. Sloma, P. C. Familletti, J. M. Tabor, M. Evinger, W. P. Levy, and S. Pestka, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7010 (1980).
[7] D. V. Goeddel, E. Yelverton, A. Ullrich, H. L. Heyneker, G. Miozzari, W. Holmes, P. H. Seeburg, T. Dull, L. May, N. Stebbing, R. Crea, S. Maeda, R. McCandliss, A. Sloma, J. M. Tabor, M. Gross, P. C. Familletti, and S. Pestka, *Nature (London)* **287**, 411 (1980).

and Ser 98 was encoded by I
(Gly 1) and the 602 bp *Pvu*II

Following ligation, the D
tated genes were inserted int
E. coli as described in this v
fermenters, harvested, and th
homogenizer.[8] Each crude ex
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washed to remove extraneou
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analogs were analyzed by SD
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Each of the three analogs
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Analysis of the purified a
cates the sulfhydryl groups at
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pattern of parental IFN-αA (l
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a "slow" monomer form only
E) appeared to be susceptible
ance of a discrete fragment w
appearance of this fragment w
traction procedure.

[8] S. J. Tamowski and R. A. Liptak, *A*
[9] T. Staehelin, D. S. Hobbs, H. Kung.
(1981).
[10] U. K. Laemmli, *Nature (London)* 22
[11] P. C. Familletti, S. Rubinstein, and

RESSION [58]

N-αA GENE FRAGMENTS*

uence

*Sau*3AI

AGCC
TCGGTCGCACTAT

GGTGGGGGTGACAG *Hin*fI
CCACCCCCACTGTCTCA

d: solid line, 9 base complementary

olecules encoded one codon
arental gene was replaced by
ed (Table I).
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and 98D (Table I) to both the
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e double substitution of Gly 1

. Familletti, J. M. Tabor, M. Evinger,
. *U.S.A.* 77, 7010 (1980).
Heyneker, G. Miozzari, W. Holmes,
Crea, S. Maeda, R. McCandliss, A.
d S. Pestka, *Nature* (*London*) 287, 411

and Ser 98 was encoded by ligation of the 273 bp *Eco*RI/*Pvu*II fragment (Gly 1) and the 602 bp *Pvu*II/*Eco*RI fragment (Ser 98).

Following ligation, the DNAs were digested with *Eco*RI and the mutated genes were inserted into the *Eco*RI site of pRC23 for expression in *E. coli* as described in this volume.[2] *E. coli* cells were grown in 10-liter fermenters, harvested, and the cell pastes were lysed in a Manton-Gaulin homogenizer.[8] Each crude extract was passed over a column of immobilized anti-leukocyte interferon monoclonal antibody.[9] The columns were washed to remove extraneous *E. coli* proteins and the analog proteins were desorbed with a dilute acetic acid solution. Samples of the purified analogs were analyzed by SDS–PAGE[10] in the presence or absence of 2-mercaptoethanol, and assayed for their ability to protect MDBK cells from a challenge by vesicular stomatitis virus *in vitro*.[11]

Each of the three analogs possessed a specific activity of 2×10^8 units/mg protein, identical to that of parental IFN-αA. This confirms that formation of the Cys 1/Cys 98 disulfide bond is not necessary for the antiviral activity of the protein. In addition, the protein analogs were purified with monoclonal antibody LI-8 indicating that Cys 1 or Cys 98 residues were not involved in the domain recognized by this immunoglobulin.

Analysis of the purified analogs by nonreducing SDS–PAGE implicates the sulfhydryl groups at positions 1 and 98 to be involved in intermolecular disulfide bond formation. As illustrated in Fig. 2, the Gly 1-substituted analog migrated almost entirely as a "slow" monomer form with some dimer form observed (lane B), compared with the migration pattern of parental IFN-αA (lane A). In contrast, the Ser 98-substituted analog possessed a much higher dimer content (lane C). This indicates that the Cys 1 sulfhydryl group is a more active participant in intermolecular disulfide bond formation than the Cys 98 sulfhydryl. That the dimer forms were disulfide bonded was demonstrated by conversion of the Cys 1/Cys 1 dimer to "slow" monomer form in the presence of 2-mercaptoethanol (lane D). The Gly 1/Ser 98 double-substituted analog migrated as a "slow" monomer form only (lane E). The "slow" monomer form (lane E) appeared to be susceptible to proteolysis as evidenced by the appearance of a discrete fragment which migrated at about 14,000 Da. The appearance of this fragment was dependent on subtle changes in the extraction procedure.

[8] S. J. Tarnowski and R. A. Liptak, *Adv. Biotechnol. Processes* 2, 271 (1983).
[9] T. Staehelin, D. S. Hobbs, H. Kung, C. Y. Lai, and S. Pestka, *J. Biol. Chem.* 256, 9750 (1981).
[10] U. K. Laemmli, *Nature* (*London*) 227, 680 (1970).
[11] P. C. Familletti, S. Rubinstein, and S. Pestka, this series, Vol. 78, p. 387.

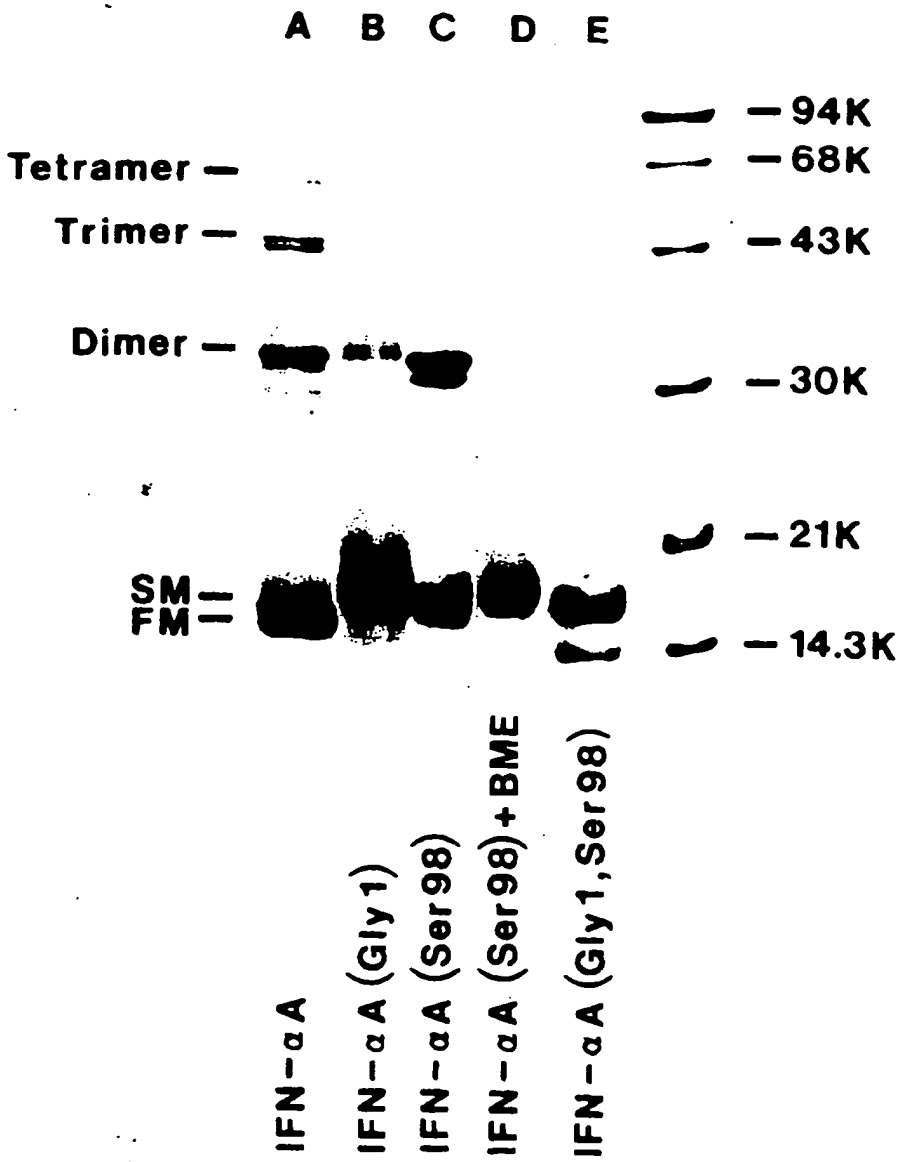

FIG. 2. Coomassie Brilliant blue-stained SDS–PAGE analysis of Cys 1/Cys 98 substituted IFN-αA analogs. Cells produced parental IFN-αA (*E. coli* W 3110 *trp* R-lac[2]/pLiFA *trp* 55), under *trp* promoter–operator control mechanisms.[7] IFN-αA (Gly 1). IFN-αA (Ser 98), or IFN-αA (Gly 1, Ser 98) (*E. coli*. RR1/pRK248cIts) were produced at 42° as described by R. Crowl.[2] Frozen cell pastes were homogenized to crude cell extracts[8] and interferon

Modification of the IFN-αA C

Mutagenesis of the IFN-α
nal 27 amino acids (139–165)
DNA heteroduplex[13] (Fig. 3).
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that mutations can be screened
with the M13 system[14]; (2) the
requirement of exonuclease II
from a nick introduced by a
amount of ethidium bromide[15];
purified from the linearized a
15% of the resulting transform
tion is a deletion (as was the c
below), or a base change to int
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Formation and Isolation of He

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linearized molecules. To obtain

[12] M. M. Bradford, *Anal. Biochem.* 72,
[13] A. Oka, K. Sugimoto, H. Sasaki, an
[14] G. Winter, A. K. Fersht, A. J. Wilkins
756 (1982).
[15] G. Dalbadie-McFarland, L. W. Cohe
Richards, *Proc. Natl. Acad. Sci. U.S*
[16] H. C. Birnboim and J. Doly, *Nucleic*

was purified on an immunosorbent colun
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analyzed by electrophoresis[10] on a 12.5
Electrophoresis of the samples under n
buffer without 2-mercaptoethanol. Lane
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IFN-αA (Ser 98), reduced; lane E. 10 μ
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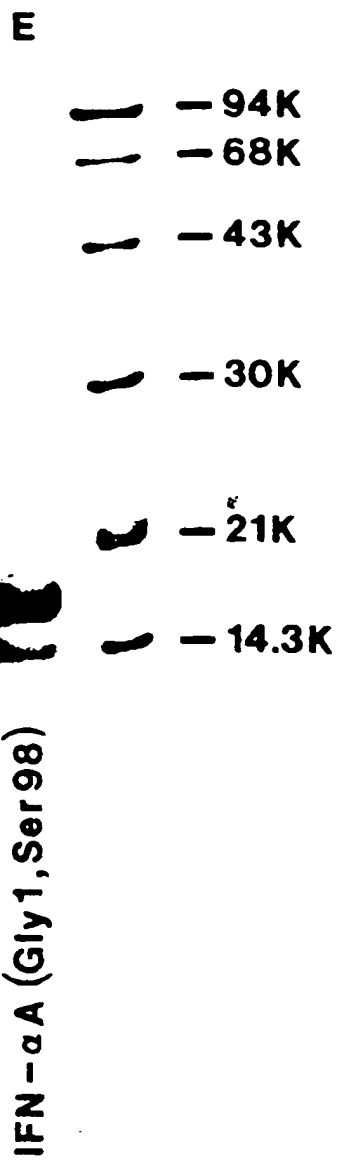
68,000, bovine serum albumin: 43,000, o
bean trypsin inhibitor; 14,300, lysozyme.
monomer with both disulfide bonds intac



AGE analysis of Cys 1/Cys 98 substi-
-αA (*E. coli* W 3110 *trp* R-lac^2/pLiFA
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Its) were produced at 42° as described
to crude cell extracts[8] and interferon

Modification of the IFN-αA Carboxy-Terminus

Mutagenesis of the IFN-αA gene region encoding the carboxy terminal 27 amino acids (139–165) was performed by generating a plasmid DNA heteroduplex[13] (Fig. 3). The advantages of this method are that (1) the mutagenesis can be performed directly on the expression plasmid so that mutations can be screened rapidly without subcloning as is necessary with the M13 system[14]; (2) the formation of heteroduplexes eliminates the requirement of exonuclease III digestion to create a single-stranded gap from a nick introduced by a restriction endonuclease and a calibrated amount of ethidium bromide[15]; and (3) since heteroduplexes can easily be purified from the linearized and gapped homoduplexes, between 5 and 15% of the resulting transformants contain mutant plasmids. If the mutation is a deletion (as was the case for IFN-αA gene mutations described below), or a base change to introduce a restriction site, then the transformants can easily be screened by a rapid plasmid isolation protocol.[16]

Formation and Isolation of Heteroduplex

Use of the three restriction enzymes (Fig. 3) generated two populations of opened plasmids: a linearized plasmid, and a linearized plasmid with a 550 bp *Pvu*II/*Bst*EII fragment removed. It is desirable that the gap be no larger than about 12% of the plasmid's size. Excessively larger gaps make it more difficult to resolve the heteroduplexes from homoduplex linearized molecules. To obtain sufficient quantities of heteroduplex, at

[12] M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).
[13] A. Oka, K. Sugimoto, H. Sasaki, and M. Takanami, *Gene* **19**, 59 (1982).
[14] G. Winter, A. K. Fersht, A. J. Wilkinson, M. Zoller, and M. Smith, *Nature (London)* **299**, 756 (1982).
[15] G. Dalbadie-McFarland, L. W. Cohen, A. D. Riggs, C. Morin, K. Itakura, and J. H. Richards, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6409 (1982).
[16] H. C. Birnboim and J. Doly, *Nucleic Acids Res.* **7**, 1513 (1979).

was purified on an immunosorbent column of immobilized monoclonal antibody LI-8.[9] Following protein determination[12] aliquots of each antibody pool were dried by evaporation and analyzed by electrophoresis[10] on a 12.5% polyacrylamide slab gel containing 0.1% SDS. Electrophoresis of the samples under nonreducing conditions was performed with sample buffer without 2-mercaptoethanol. Lane A, 15 μg parental IFN-αA, nonreduced; lane B, 10 μg IFN-αA (Gly 1), nonreduced; lane C, 10 μg IFN-αA (Ser 98), nonreduced; lane D, 10 μg IFN-αA (Ser 98), reduced; lane E, 10 μg IFN-αA (Gly 1, Ser 98), nonreduced. Standard protein makers from Bio-Rad Laboratories (Rockville, NY): 94,000, phosphorylase *b*; 68,000, bovine serum albumin; 43,000, ovalbumin; 30,000, carbonic anhydrase; 21,000, soybean trypsin inhibitor; 14,300, lysozyme, all reduced. SM, "slow" monomer; FM, "fast" monomer with both disulfide bonds intact.

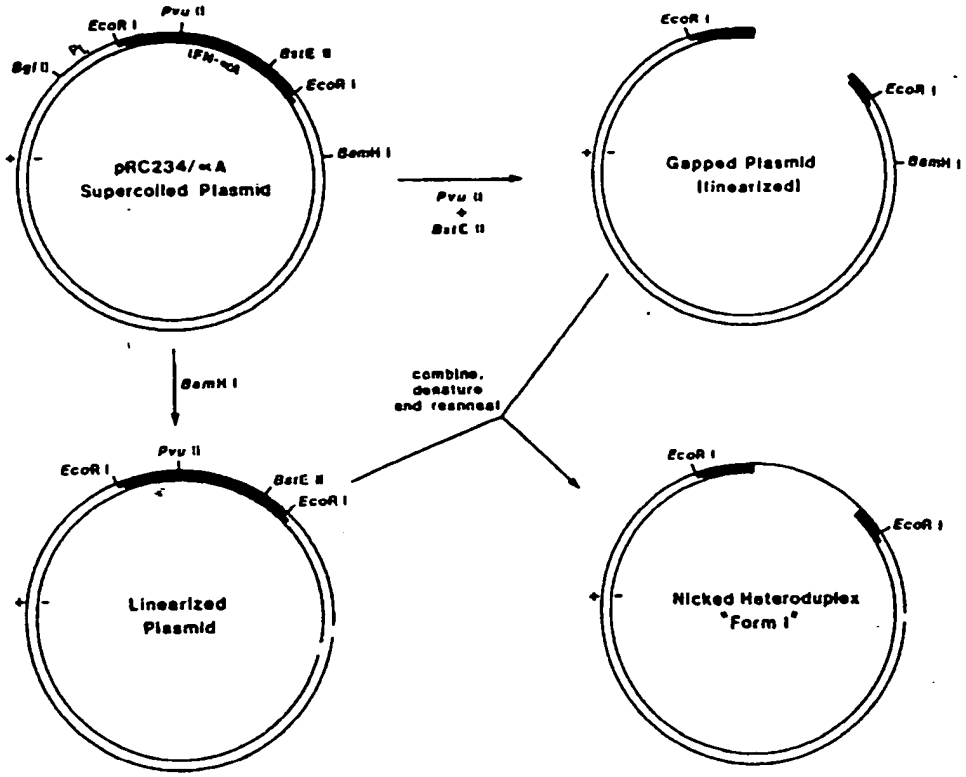

FIG. 3. Formation of plasmid DNA heteroduplex as a base for site-directed mutagenesis. See text for details. pRC 234 is a derivative of pRC 23 with the *Pvu*II site in the plasmid deleted.

least 1 μg of supercoiled plasmid was used for each of the two restriction enzyme digests. After digestion, the linearized plasmids were purified by electrophoresis through agarose, recovered, and precipitated with ethanol.

Purified linearized plasmids were resuspended in 25 μl of H_2O and combined in an Eppendorf tube. After addition of 50 μl of a solution containing 0.2 *N* NaOH and 40 m*M* EDTA, the plasmids were denatured at 23°. After 10 min, 10 μl of 2 *M* Tris (1.8 *M* Tris · HCl, 0.2 *M* Tris base) and 110 μl of deionized formamide were added. Annealling of single-stranded DNA proceeded for about 3 hr at 23°. Two heteroduplex forms resulted during the annealling process, Form I from the annealing of the "+" strand of the linearized plasmid with the "−" strand of the gapped

plasmid, and Form II from
single-stranded gap of 550
desired mutations on the
necessary to separate the
gonucleotides were compler

After annealling, 300 μl o
were added and the heterod
with ethanol. After the seco
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To recover the heteroduplex
tion. The gel strip containi
dialysis bag with 1 ml of 1
bromide (50 μg/ml). The he
long wavelength UV as they
After electrophoresis, the e
with four 1 ml rinses of the
m*M* Tris · HCl, pH 7.4, 1 *M*
used to reduce the volume of
added and the heteroduplexe
chloroform/isoamyl alcohol
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Introduction of Deletions in

Deletions in plasmid DN
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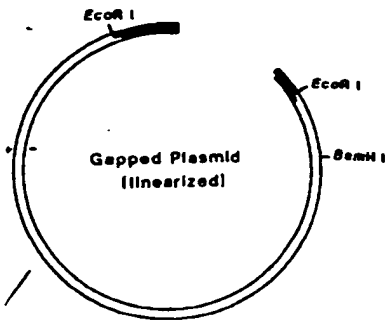

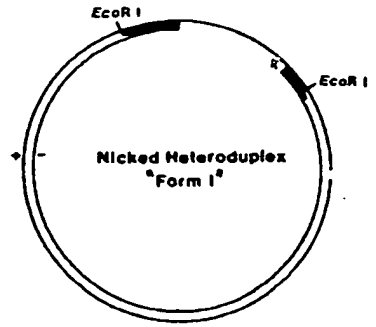

a base for site-directed mutagenesis.
) with the *Pvu*II site in the plasmid

or each of the two restriction
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M Tris · HCl, 0.2 *M* Tris base)
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23°. Two heteroduplex forms
n I from the annealing of the
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plasmid, and Form II from annealling of the other strands. In Form I, a single-stranded gap of 550 nucleotides was created in the region of the desired mutations on the "+" strand in the IFN-αA gene. It was not necessary to separate the two heteroduplex forms since the deoxyoligonucleotides were complementary to the "+" strand only.

After annealling, 300 μl of 0.3 NaAc, pH 7.0 and 20 μg of carrier tRNA were added and the heteroduplex molecules were precipitated two times with ethanol. After the second centrifugation, the pellet was rinsed with cold ethanol and dried under vacuum. The heteroduplex forms were purified by electrophoresis through a 0.7% agarose gel. The heteroduplex forms migrated with nicked parental plasmid, but slower than both linearized forms.

To recover the heteroduplex forms, it was necessary to use electroelution. The gel strip containing the heteroduplex band was placed in a dialysis bag with 1 ml of TAE, pH 7.8, running buffer with ethidium bromide (50 μg/ml). The heteroduplex molecules were visualized under long wavelength UV as they migrated out of the gel strip into the buffer. After electrophoresis, the elution buffer was recovered and combined with four 1 ml rinses of the dialysis bag with a solution consisting of 10 m*M* Tris · HCl, pH 7.4, 1 *M* EDTA, and 300 m*M* NaCl. 2-Butanol was used to reduce the volume of 0.5 ml. Ten micrograms of carrier tRNA was added and the heteroduplexes were extracted once with 500 μl of phenol/chloroform/isoamyl alcohol (50:50:1), precipitated two times with ethanol, dried, and resuspended in 3 μl of H_2O. About 50 ng of heteroduplexes (as measured by visual comparison with standardized plasmid DNA on an agarose gel) was recovered from the gel. Electroelution from agarose was necessary due to the strong affinity between the single-stranded region of the heteroduplex and agarose. It was also important not to filter the eluted heteroduplex through nitrocellulose because single-stranded DNA has high affinity for the filter.

Introduction of Deletions in IFN-αA with Synthetic DNA

Deletions in plasmid DNA were used to produce truncated IFN-αA proteins which were terminated after Cys 138, Val 143, or Arg 149 (Fig. 1a). The three deoxyoligonucleotides shown in Table II each directed a loop-out of sequences in the "+" strand to position the TGA stop codon immediately downstream of the sequences encoding Cys 138, Val 143, or Arg 149. By designing the synthetic DNAs to complement 12 nucleotides in the "+" strand on the 5′ side of the splice point and 12 nucleotides on the 3′ side, the intervening sequences not complementary to the deoxyoligonucleotide will loop-out as illustrated in Fig. 4.

TABLE II

SYNTHETIC DNA FOR INTRODUCING DELETIONS INTO IFN-αA[a]

					138					143						149			166				
Parental	Lys	Tyr	Ser	Pro	Cys	Ala	Trp	Glu	Val	Val	Arg	Ala	Glu	Ile	Met	Arg	Ser		Glu	END			
"+" 5'. . .	.AAA	TAC	AGC	CCT	TGT	GCC	TGG	GAG	GTT	GTC	AGA	GCA	GAA	ATC	ATG	AGA	TCT	. . .	.GAA	TGA	AAA	CTG	GTT
					Cys	END																	
Δ138 3'		ATG	TCG	GGA	ACA	ACT	TTT	GAC	CAA														
										Val	END												
Δ143 3'							ACC	CTC	CAA	CAG	ACT	TTT	GAC	CAA									
																Arg	END						
Δ149 3'													CTT	TAG	TAC	TCT	ACT	TTT	GAC	CAA			

[a] Δ138 directs a loop-out of 81 bases; Δ143 directs a loop-out of 66 bases; Δ149 directs a loop-out of 48 bases.

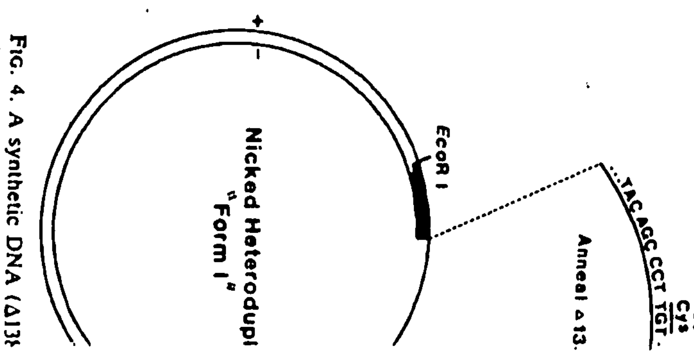

FIG. 4. A synthetic DNA (Δ13
terminal coding sequences. In this e
IFN-αA sequences not complemen
results from the replication of the "

To effect mutagenesis, th
"+" strand in the Form I het
50-fold molar excess of pho
bined with the purified heter
7.4. The mixture was heated
cooling prevented the compl
teroduplex forms from self-a
eral minutes at 0°, the volum
mM Tris · HCl, pH 7.4, 50 m
ATP, 400 μM of each of the
of Klenow fragment of E. co
DNA Ligase. Filling-in of the
tion was performed at 15° for

[17] R. B. Wallace, M. Schold, M. J. Jo
9, 3647 (1981).

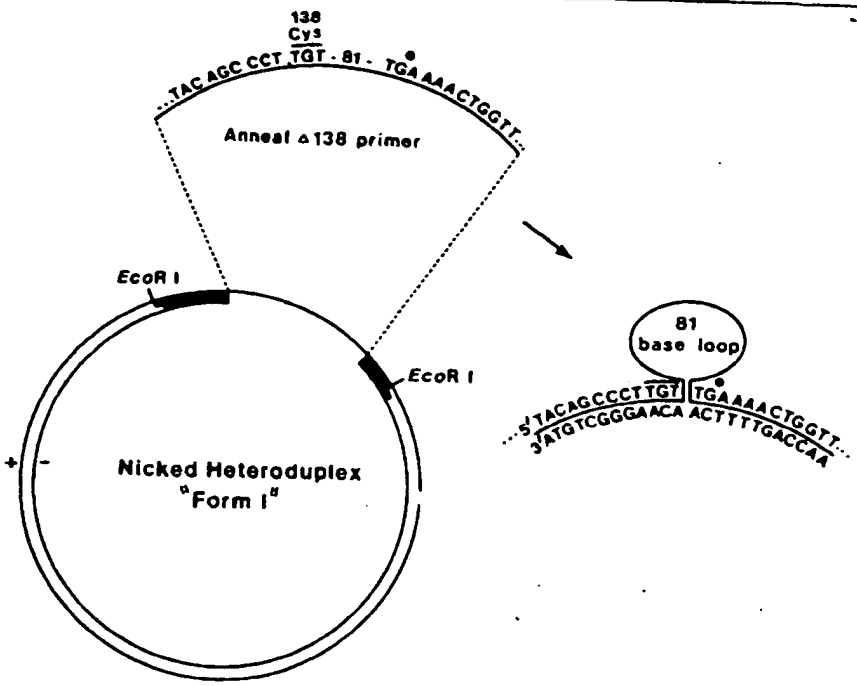

FIG. 4. A synthetic DNA (Δ138) directs the precise splicing of the IFN-αA carboxy-terminal coding sequences. In this example, a deletion of 81 bp resulted from a loop-out of IFN-αA sequences not complementary to the deoxyoligonucleotide. The mutant plasmid results from the replication of the "−" strand.

To effect mutagenesis, the deoxyoligonucleotide was annealled to the "+" strand in the Form I heteroduplex as has been described.[17] Briefly, a 50-fold molar excess of phosphorylated deoxyoligonucleotide was combined with the purified heteroduplex in a volume of 5 μl of 0.1 × TE, pH 7.4. The mixture was heated at 68° for 3 min and quenched at 0°. The rapid cooling prevented the complementary single-stranded regions of both heteroduplex forms from self-annealling to displace the primer. After several minutes at 0°, the volume was adjusted to 20 μl by the addition of 10 m*M* Tris · HCl, pH 7.4, 50 m*M* NaCl, 100 m*M* $MgCl_2$, 1 *M* DTT, 400 μ*M* ATP, 400 μ*M* of each of the four deoxynucleotide triphosphates, 3 units of Klenow fragment of *E. coli* DNA Polymerase I, and 120 units of T_4-DNA Ligase. Filling-in of the single-stranded region and subsequent ligation was performed at 15° for 6 hr. The reaction volume was then adjusted

[17] R. B. Wallace, M. Schold, M. J. Johnson, P. Dembek, and K. Itakura, *Nucleic Acids Res.* 9, 3647 (1981).

Val END
Δ143 3' ACC CTC CAA CAG ACT TTT GAC CAA

Arg END
Δ149 3' CTT TAG TAC TCT ACT TTT GAC CAA

• Δ138 directs a loop-out of 81 bases; Δ143 directs a loop-out of 66 bases; Δ149 directs a loop-out of 48 bases.

to 250 μl by the addition of 0.3 *M* sodium acetate, pH 7.0, and extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1). The DNA was precipitated twice with ethanol, dried, and resuspended in 5 μl of H_2O for transformation of *E. coli* MC1061.[18] The efficiency of obtaining a mutation was about 5–15% of the colonies screened. Each positive colony detected by mini-prep plasmid preparation had two plasmids present, one plasmid with the deletion and the parental plasmid. Thus, it was necessary to retransform with plasmid DNA to select those colonies which contain the plasmid with the deletion. Similarly, analogs of IFN-αA prematurely terminated at Val 143 and Arg 149 were prepared.

Analysis of Prematurely Terminated IFN-αA Proteins

The results obtained above for the Cys 1 and Cys 98 substitutions were indirect evidence for the importance of Cys 29/Cys 138 intramolecular disulfide bond formation since fully reduced IFN-αA retains only 5% of its activity in the MDBK cell assay.[19] Therefore, it was interesting to determine if amino acids distal to Cys 138 were necessary for biological activity.

E. coli cells which contained plasmids with the specific deletions were induced at 42° and extracts were prepared by 7 *M* guanidine hydrochloride lysis as described.[2] Each extract demonstrated no biological activity in the MDBK cell assay. To determine whether these analogs were inactive as the direct result of the carboxy-terminal deletions or because of *in vivo* instability, plasmids encoding the Cys 138, Val 143, Arg 149, and parental IFN-αA proteins were used in a cell-free, prokaryotic DNA-dependent transcription/translation system.[20] The IFN-αA analogs were synthesized to a level at least equal to that of parental IFN-αA, but were biologically inactive. This result suggests that amino acids distal to the Arg residue at position 149 in the primary structure are required for maximal antiviral activity of IFN-αA.

Concluding Comments

This chapter illustrates two approaches for mutagenesis. The approach used for generating the Cys 1 and Cys 98 substitutions requires extensive synthesis of deoxyoligonucleotide duplexes. The heteroduplex

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method requires only a si
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tance between restriction s
12% of the plasmid length.
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Deletions made in the
provided evidence that am
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and Arg[149] were assayed, th
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cells. Zoon *et al.*[22] report
thermolysin digestion conta
exhibited about 1/100th of
cells. Recently, Nisbet *et al*
in IFN-αI that showed signi
It was reported by Levy *et*
interferon closely correspo
position 155, 10 amino acid
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carboxy-terminal 10–13 am
IFN-αA activity.[25,26] This i
presented here, suggests tha
boxy-terminal region (i.e., a
quired for maximal antiviral

Acknowledgments

We would like to thank Drs. F
Pennina Langer-Safer for helpful dis
tions of *E. coli* cells. Many thanks
manuscript.

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Gerber, S. Stein, and S. Pestka, *P.*
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[26] N. Chang, H.-F. Kung, and S. Pes

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. 138, 179 (1980).

'ol. Chem. 250, 1556 (1975).

method requires only a single synthetic deoxyoligonucleotide and mutagenesis can be performed directly on the expression plasmid. The distance between restriction sites used for making the gap is limited to about 12% of the plasmid length. While this work was in progress, an improvement of the heteroduplex method was reported.[21]

Deletions made in the IFN-αA gene carboxy-terminal coding region provided evidence that amino acid residues distal to Arg^{149} are required for full IFN-αA activity when measured on MDBK cells. When purified preparations (data not shown) of IFN-αA molecules terminated at Cys^{138} and Arg^{149} were assayed, they showed approximately 1/100th of the activity of a molecule terminated at Phe^{151} and full length IFN-αA on MDBK cells. Zoon *et al.*[22] reported that a fragment of IFN-αA produced by thermolysin digestion containing the amino-terminal 110 amino acids also exhibited about 1/100th of the activity of the intact molecule on MDBK cells. Recently, Nisbet *et al.*[23] made a glycine substitution for tyrosine 136 in IFN-α1 that showed significant loss of antiviral activity on bovine cells. It was reported by Levy *et al.*[3,24] that two natural species of leukocyte interferon closely corresponding to IFN-αA in sequence terminated at position 155, 10 amino acids shorter than IFN-αA. These species were fully active interferon molecules. In addition, it has been reported that the carboxy-terminal 10–13 amino acids can be eliminated without loss of IFN-αA activity.[25,26] This information, taken together with the results presented here, suggests that structural features contributed by the carboxy-terminal region (i.e., amino acids 151–154) of IFN-αA may be required for maximal antiviral activity on MDBK cells.

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